NEW AGENTS FOR TRANSFERRING NUCLEIC ACIDS, COMPOSITIONS CONTAINING THEM AND THEIR USES

The present invention relates to new compounds which are useful as agents for transferring nucleic acids into cells. These new compounds are more particularly related to the lipopolyamine family and comprise at least one cyclic amidine function. They are useful for transfecting nucleic acids to various types of cells, *in vitro*, *ex vivo* or *in vivo*.

With the development of biotechnology, the possibility of effectively transferring nucleic acids into cells has become a necessity. It may involve the transfer of nucleic acids into cells *in vitro*, for example for the production of recombinant proteins, or in the laboratory for studying the regulation of the expression of genes, the cloning of genes, or any other manipulation involving DNA. It may also involve the transfer of nucleic acids into cells *in vivo*, for example for the creation of transgenic animals, the production of vaccines, carrying out labelling studies or also therapeutic approaches. It may also involve the transfer of nucleic acids into cells *ex vivo*, into approaches for bone marrow grafts, immunotherapy or other methods involving the transfer of genes into cells collected from an organism for the purpose of their subsequent readministration.

Various types of synthetic vectors have been developed in order to improve the transfer of nucleic acid into cells. Among these vectors, cationic lipids possess advantageous properties. These vectors consist of a cationic polar portion, which interacts with the nucleic acids, and of a hydrophobic lipid portion which favours cell penetration. Specific examples of cationic lipids are in particular the monocationic lipids (DOTMA : Lipofectin®), some cationic detergents (DDAB), lipopolyamines and in particular dioctadecylamidoglycyl spermine (DOGS) or palmitoylphosphatidylethanolamine 5-carboxyspermylamide (DPPES), whose preparation has been described, for example, in patent application EP 394,111. Another lipopolyamine family is represented by the compounds as described in patent application WO 97/18185 incorporated into the present application by way of reference, and are illustrated in Figure 1.

However, up until now, injections into tissues, in particular the muscles, were often made with non-formulated DNA in order to facilitate its entry into the cells, the combination with synthetic vectors leading to complexes which are too large in size to be incorporated into the cells.

It is one of the main problems which the present invention proposes to solve. Indeed, compounds according to the invention possess the unexpected advantage of having a level of transfection *in vivo* into the muscle which is at least equivalent to that

obtained with non-formulated DNA and in any case a very good level of transfection into the other tissues. The combination with a compound according to the invention protects the DNA from degradations by nucleases and/or from deteriorations during freeze-drying, which contributes towards significantly improving the stability of the nucleolipid formulations. Furthermore, such a combination allows a slow controlled release of the nucleic acids.

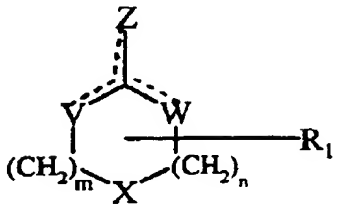
Moreover, the compounds according to the present invention belong to the family of cationic lipids and carry a novel cationic region which confers improved properties on said compounds, in particular reduced cytotoxicity compared with the prior art cationic vectors. This cationic portion is indeed more precisely represented by one or more particular polyamine(s), carrying one or more cyclic amidine functions which very probably have the effect of "delocalizing" the positive charges, making the compound less cationic overall, with the resulting beneficial effects known the toxicity point of view.

Thus, a first subject of the invention relates to new compounds, in D, L or DL form, of general formula (I):

$$CA—Rep—R \qquad (I)$$

for which:

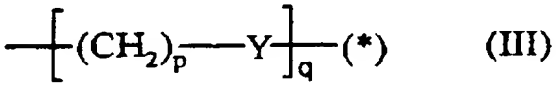
① CA represents a cycloamidine group and its mesomeric forms of general formula (II):



(II)

for which:

- m and n are integers, independent of each other, of between 0 and 3 inclusive and such that m+n is greater than or equal to 1,
- R_1 represents a group of general formula (III):

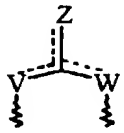


(III)

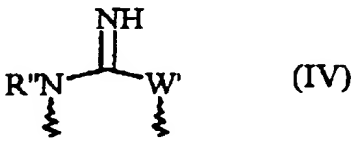
for which p and q are integers, independent of each other, of between 0 and 10 inclusive, Y represents a carbonyl, amino, methylamino or methylene group, it being possible for Y to have different meanings within the different groups $[(CH_2)_p\text{-}Y]$, and (*) represents either a hydrogen atom or is the site for bonding to the group Rep,

it being understood that R_1 may be bonded to any atom of general formula (II), including Z, and that there is a single group R_1 in formula (II),

- X represents a group NR_2 or CHR_2, R_2 being either a hydrogen atom or the bonding to the group R_1 as defined above,

- The group  represents:

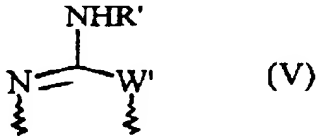
*1st case: a group of general formula (IV):



(IV)

for which W' represents CHR□ or NR□, and R" and R□ represent, independently of each other, a hydrogen atom, a methyl, or the bonding to the group R_1 as defined above, or

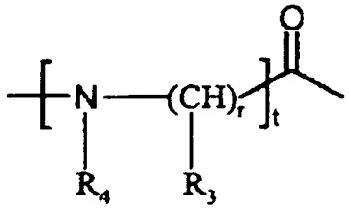
*2nd case: a group of general formula (V):



(V)

for which W' represents CHR□ or NR□, and R' and R□ represent, independently of each other, a hydrogen atom, a methyl or the bonding to the group R_1 as defined above,

② Rep is absent or is a spacer of general formula (VI):



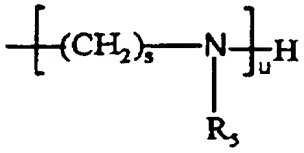
(VI)

whose nitrogen atom is attached to the atoms X, V, W or Z or to the substituent Y of the group R_1 depending on the cases, and

- t is an integer between 0 and 8 inclusive,

• r is an integer between 0 and 10 inclusive, it being possible for r to have different meanings within the different groups $-NR_4-(CH)_r-$,

• R_3, which may have different meanings within the different groups $NR_4-(CH)_rR_3$, represents a hydrogen atom, a methyl group or a group of general formula (VII):



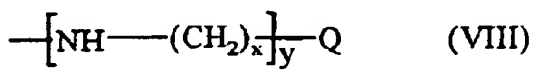
(VII)

for which u is an integer between 1 and 10 inclusive, s is an integer between 2 and 8 inclusive which may have different meanings within the different groups $-(CH_2)_s-NR_5$, and R_5 is a hydrogen atom, a group CA as defined above, it being understood that the groups CA are independent from each other and may be different, or a group of general formula (VII), it being understood that the groups of general formula (VII) are independent of each other and may have different meanings,

• R_4 is defined in the same manner as R_3 or represents a group CA as defined above, it being understood that the groups CA are independent of each other and may be different, and

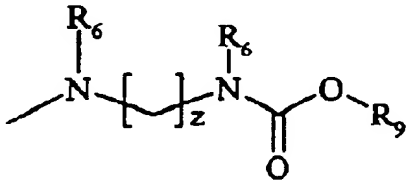
③ R is bonded to the carbonyl function of the group Rep of general formula (VI), or if Rep is absent, R is bonded directly to the group CA, and represents:

* either a group of formula NR_6R_7 for which R_6 and R_7 represent, independently of each other, a hydrogen atom or an optionally fluorinated, linear or branched, saturated or unsaturated aliphatic radical containing 1 to 22 carbon atoms, with at least one of the two substituents R_6 or R_7 different from hydrogen and the other containing between 10 and 22 carbon atoms,
* or a steroid derivative,
* or a group of general formula (VIII):



(VIII)

for which x is an integer between 1 and 8 inclusive, y is an integer between 1 and 10 inclusive, and either Q represents a group $C(O)NR_6R_7$ for which R_6 and R_7 are as defined above, or Q represents a group $C(O)R_8$ for which R_8 represents a group of formula (IX):



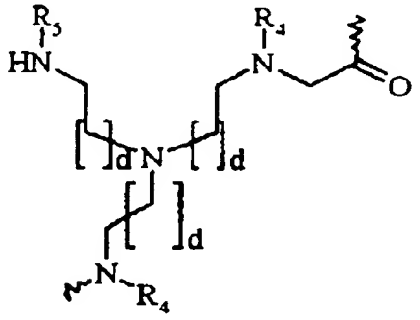
(IX)

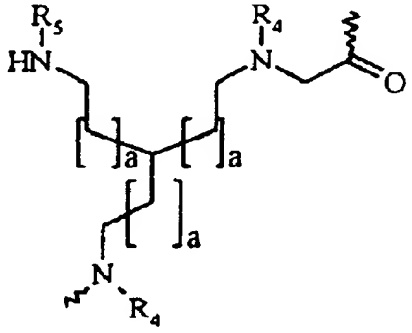
for which z is an integer between 2 and 8 inclusive, and R_9 is an optionally fluorinated, saturated or unsaturated aliphatic radical containing 8 to 22 carbon atoms, or a steroid derivative, and the two substituents R_6 are, independently of each other, as defined above,
or R_8 represents a group $-O-R_9$ for which R_9 is as defined above.

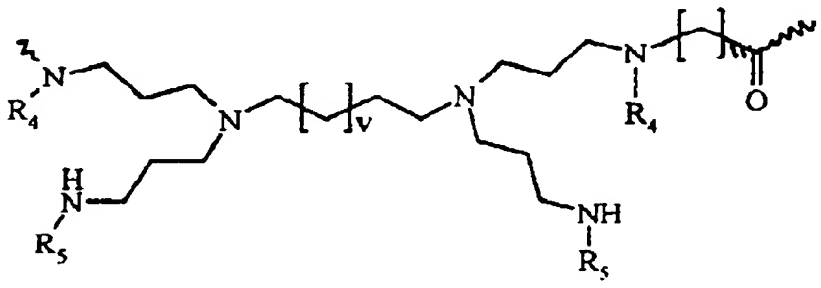
According to one variant of the invention, the group R_1 is bonded either to Z or to V, on the one hand, and to the group Rep, on the other hand, via Y.

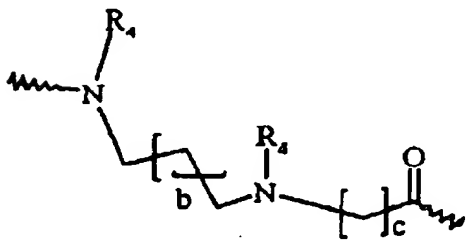
Advantageously, the cycloamidine group CA of formula (II) comprises 5, 6, 7 or 8 members.

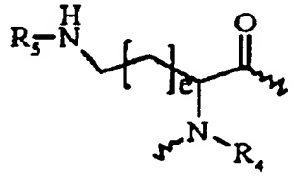
Moreover, in another variant of the invention, Rep is a spacer with 1, 2 or 3 "arms". The following spacers may for example be mentioned:











According to a second variant of the invention, R_3 represents a hydrogen atom or a methyl and R_4 is as defined above, or R_3 and R_4 present in

formula (VI) represent hydrogen atoms, or R_4 is a hydrogen atom and R_3 is a group of formula (VII) in which R_5 represents a group CA.

Preferably, in formula (V), p and q are chosen, independently of each other, from 2, 3 or 4.

In general, the group R contains at least one hydrophobic segment. For the purposes of the invention, "hydrophobic segment" is understood to mean any group of the lipid type, which promotes cell penetration. In particular, the group R contains at least one aliphatic chain or at least one steroid derivative.

According to a preferred variant, the group R represents a group of formula NR_6R_7, R_6 and R_7 being as defined above, or represents a group of general formula (VIII) in which Q represents a group $C(O)NR_6R_7$, R_6 and R_7 being as defined above.

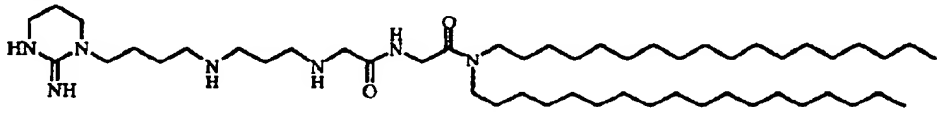
Preferably, R_6 and/or R_7 represent, independently of each other, a saturated or unsaturated linear aliphatic chain containing 10 to 22 carbon atoms, preferably with 12, 14, 16, 17, 18 or 19 carbon atoms. They are, for example, $(CH_2)_{11}CH_3$, $(CH_2)_{13}CH_3$, $(CH_2)_{15}CH_3$, $(CH_2)_{17}CH_3$ or oleyl groups and the like.

In a specific embodiment, the groups R_6 and R_7 are identical or different and each represent an optionally fluorinated, linear or branched, saturated or unsaturated aliphatic chain containing 10 to 22 carbon atoms, as defined in the preceding paragraph.

When R represents a steroid derivative, the latter is advantageously chosen from cholesterol, cholestanol, 3-α-5-cyclo-5-α-cholestan-6-β-ol, cholic acid, cholesteryl formate, chotestanyl formate, 3α,5-cyclo-5α-cholestan-6β-yl formate, cholesterylamine, 6-(1,5-dimethylhexyl)-3a,5a-dimethyl-hexadecahydrocyclopenta[a]cyclopropa[2,3]cyclo-penta[1,2-f]naphthalen-10-ylamine or cholestanylamine.

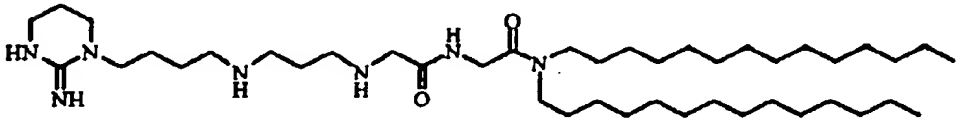
These new compounds of general formula (I) may be provided in the form of nontoxic and pharmaceutically acceptable salts. These nontoxic salts comprise salts with inorganic acids (hydrochloric, sulphuric, hydrobromic, phosphoric or nitric acids) or with organic acids (acetic, propionic, succinic, maleic, hydroxymaleic, benzoic, fumaric, methanesulphonic or oxalic acids) or with inorganic bases (sodium hydroxide, potassium hydroxide, lithium hydroxide or calcium hydroxide) or with organic bases (tertiary amines such as triethylamine, piperidine or benzylamine).

By way of example illustrating the preferred compounds according to the invention, the compounds of the following formulae may be mentioned:



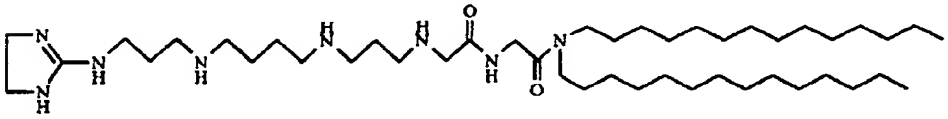
Compound (1)

N-dioctadecylcarbamoylmethyl-2-{3-[4-(2-iminotetra-hydropyrimidin-1-yl)butylamino]propylamino}acetamide



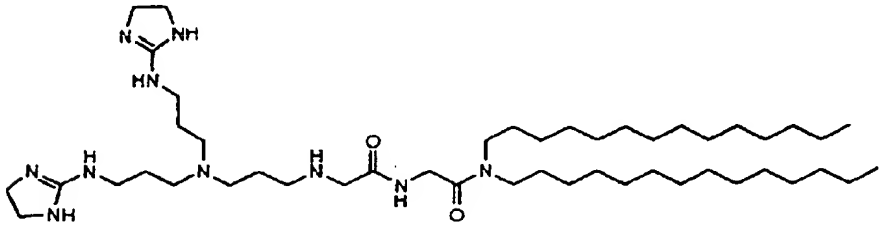
Compound (2)

N-ditetradecylcarbamoylmethyl-2-{3-[4-(2-iminotetra-hydropyrimidin-1-yl)butylamino]propylamino}acetamide



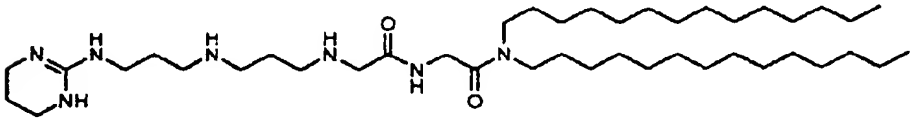
Compound (3)

2-(3-{4-[3-(4,5-dihydro-1H-imidazol-2-ylamino)-propylamino]butylamino}-N-ditetradecylcarbamoylmethyl-acetamide



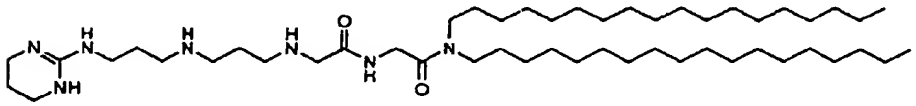
Compound (4)

2-(3-{bis[3-(4,5-dihydro-1H-imidazol-2-ylamino)propyl]-amino}propylamino)-N-ditetradecylcarbamoylmethyl-acetamide



Compound (5)

N-ditetradecylcarbamoylmethyl-2-{3-[3-(1,4,5,6-tetra-hydropyrimidin-2-ylamino)propylamino]propylamino}-acetamide



Compound (6)

N-dioctadecylcarbamoylmethyl-2-{3-[3-(1,4,5,6-tetra-hydropyrimidin-2-ylamino)propylamino]propylamino}-acetamide

The compounds of the invention may be prepared in various ways. According to a first method, the compounds of the invention may be obtained by synthesis of analogous lipopolyamines (that is to say the same structure but with no cycloamidine group), the cyclization to cycloamidine groups being carried out in a second instance. The analogous lipopolyamines may be obtained by any method known to persons skilled in the art, and in particular according to the methods described in Application WO 97/18185 or by similar methods. The cyclization of the amidine heads may, for example, be carried out by reaction between one and/or more primary amines of the lipopolyamine and reagents such as O-methylisourea sulphate hydrogen sulphate [J. Med. Chem., 1995, 38(16), pp. 3053-3061] or S-methylisothiourea hemisulphate [Int. J. Pept. Prot. Res., 1992, 40, pp. 119-126]. Preferably, the procedure is carried out in aqueous medium in the presence of a

base in the hot state [J. Med. Chem. 1985, pp. 694-698 and J. Med. Chem., 1996, pp. 669-672]. As preferred solvents, there may be mentioned water/alcohol mixtures or dimethylformamide. As base, triethylamine, N-ethyldiisopropylamine, sodium hydroxide, potassium hydroxide and the like may be used. The temperature is preferably between 40°C and 60°C, and still more preferably the reaction is carried out at 50°C.

Another method consists in carrying out a synthesis of building blocks carrying the cycloamidine function which are then grafted onto lipids equipped with spacers. This method has the advantage of providing access to a large number of products. For the purposes of the invention, "blocks" is understood to mean any functional segment of the molecule. For example, the cycloamidine group CA as defined in general formula (II), Rep or R each constitute distinct blocks for the purposes of the invention.

By way of example, the procedure may, for example, be carried out in the following manner:

1) Synthesis of the building block R:

a) When R represents $-NR_6R_7$, either it is commercially available, or it can be synthesized according to one of the following methods:

- by alkylative reduction between an amine carrying the group R_6 and an aldehyde carrying the group R_7. The procedure is preferably carried out in a chlorinated solvent (for example dichloromethane, chloroform,

1,2-dichloroethane and the like [J. Org. Chem., 1996, pp. 3849-3862]) or in any other organic solvent which is compatible with the reaction (for example tetrahydrofuran), in the presence of sodium triacetoxyborohydride, sodium cyanoborohydride or derivatives thereof (for example lithium cyanoborohydride) [J. Am. Chem. Soc., 1971, pp. 2897-2904] and acetic acid.

- or by substitution of a leaving group carried by R_6, by an amine carrying the group R_7. By way of example of a leaving group, halogen atoms (Br, Cl, I) or tosyl or mesyl substituents and the like may be mentioned. The procedure is preferably carried out in the presence of a basic reagent, for example sodium carbonate, potassium hydroxide, sodium hydroxide, triethylamine and the like, in an alcohol (for example ethanol) under reflux [J. Am. Chem. Soc., 1996, pp. 8524-8530].
- or by coupling between a fatty acid (or derivatives thereof such as fatty acid chlorides) and a fatty amine. The amide obtained is then reduced by a hydride, for example lithium aluminium hydride or any other hydride known to persons skilled in the art, in an ether (for example tetrahydrofuran (THF), *t*-butyl methyl ether (TBME), dimethoxyethane (DME) and the like).

b) When R represents a group of general formula (VIII), the peptide coupling is carried out between the group Q and H-[NH-$(CH_2)_x$]$_y$COOH. The peptide coupling is

carried out according to conventional methods known to persons skilled in the art (Bodanski M., *Principles and Practices of peptide Synthesis*, Ed. Springe-Verlag) or by any known similar method. In particular, the reaction may be carried out in the presence of a nonnucleophilic base in suitable aprotic solvents (such as chloroform, dimethylformamide, methylpyrrolidone, acetonitrile, dichloromethane and the like), at a temperature of between 0 and 100°C, the pH being adjusted between 9 and 11.

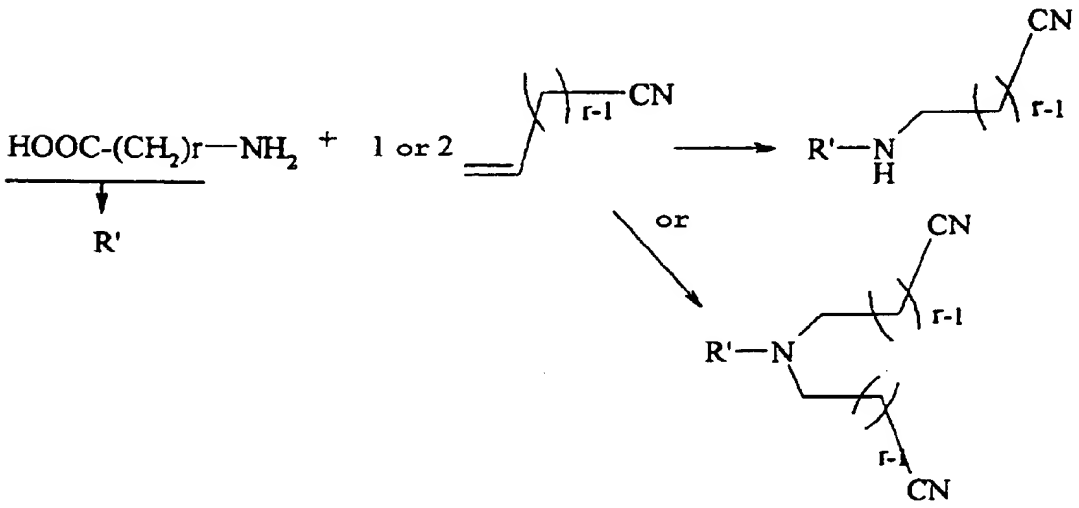
Q is either commercially available or, when Q represents a group C(O)R8 with R8 of formula (IX), it may be synthesized by reaction between a chloroformate which is commercially available (for example cholesteryl chloroformate) or obtained according to conventional methods known to persons skilled in the art from a commercially available chloroformate, and a diamine which is commercially available (for example N-ethylenediamine) or obtained according to conventional methods known to persons skilled in the art. Preferably, the procedure is carried out in a chlorinated solvent (for example dichloromethane, chloroform, 1,2-dichloroethane and the like) or in any other organic solvent which is compatible with the reaction, such as for example dimethylformamide, dimethyl sulphoxide, acetonitrile and the like.

The group H-[NH-(CH2)x]y-COOH is a commercially available amino acid when y is equal to 1, or is obtained by one or more cyanoethylation reactions according to the method described below in the synthesis of Rep when y is greater than 1.

2) Synthesis of the building block Rep:

The group Rep is obtained by cyanoethylation or by dicyanoethylation (depending on whether it is desired to obtain a linear or branched Rep structure) of an amino acid of formula $HOOC-(CH_2)_r-NH_2$ and then by reduction of the nitrile functions into amines.

a) Mono- or dicyanoethylation:



Preferably, the procedure is carried out in a basic aqueous medium. For example, the reaction is carried out in solvents such as water, alcohols (for example methanol, ethanol and the like), in the presence of a base such as sodium hydroxide, potassium hydroxide, triethylamine and the like. In the case of monocyanoethylation, the work is preferably carried out

in the cold state [J. Am. Chem. Soc., 1950, pp. 2599-2603]. In the case of dicyanoethylation, the work is preferably carried out in the hot state and with an excess of acrylonitrile [J. Am. Chem. Soc., 1951, pp. 1641-1644].

b) The reduction of the nitrile functions into amines is carried out by catalytic hydrogenation in a basic medium or by any other method known to a person skilled in the art. By way of example, it is possible to use platinum oxide or Raney nickel [J. Org. Chem., 1988, pp. 3108-3111] as catalyst. Preferably, the solvent chosen is an alcohol (for example methanol, ethanol and the like) in the presence of a base, for example sodium hydroxide, potassium hydroxide and the like.

3) Synthesis of the building block Rep-R:

The building block Rep-R is obtained by peptide coupling between the acid Rep and the amine R which are obtained in the preceding steps.

The peptide coupling is carried out according to conventional methods known to persons skilled in the art (Bodanski M., *Principles and Practices of peptide Synthesis*, Ed. Springe-Verlag) or by any known similar method. In particular, the reaction may be carried out in the presence of a nonnucleophilic base, in suitable aprotic solvents (such as chloroform, dimethylformamide, methylpyrrolidione, acetonitrile, dichloromethane and the like), at a temperature of

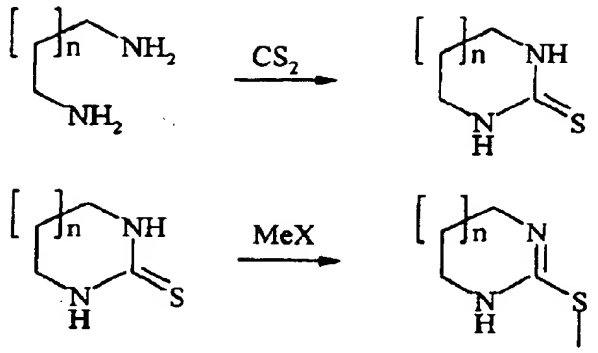
between 0 and 100°C, the pH being adjusted between 9 and 11.

4) Synthesis of the compounds according to the invention CA-Rep-R:

The compounds according to the invention are obtained according to several possible methods:

a) By coupling in a basic medium between the terminal amine present on Rep-R obtained in the preceding step, and CA-S-CH_3, according to conventional methods known to persons skilled in the art. The procedure is preferably carried out in a chlorinated solvent (for example dichloromethane, chloroform and the like) or in other organic solvents compatible with the reaction, such as for example water, alcohols, dimethylformamide and the like, in the presence of a base (for example triethylamine, sodium hydroxide, potassium hydroxide, N-ethyldiisopropylamine and the like), and at room temperature (about 20°C).

The building block CA-S-CH_3 is either commercially available (that is the case for example for 2-methylthio-2-imidazoline hydriodide), or it can be obtained by the action of a carbon disulphide on an appropriate diamine (that is to say chosen as a function of the cycloamidine group which it is desired to obtain), followed by a methylation. For example, the reaction scheme may be illustrated in the following manner:



Preferably, the reaction process is carried out in an alcohol (for example ethanol). The methylation step is carried out by the action of a halomethyl, it being possible for the halogen atom to be, for example, an iodine atom [J. Am. Cem. Soc., 1956, pp. 1618-1620 and Bioorg. Med. Chem. Lett., 1994, pp. 351-354].

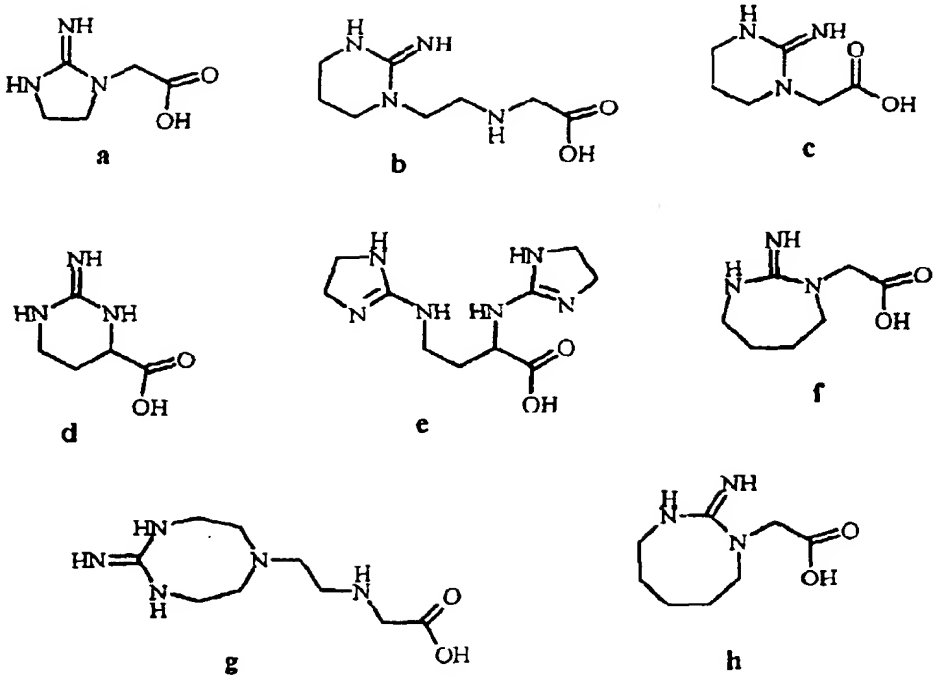
b) By internal cyclization of the cycloamidine group from the amino functions present on Rep-R, by the action of O-methylisourea hydrogen sulphate or S-methylisothiourea hemisulphate. Preferably, the procedure is carried out in an aqueous medium in the presence of a base in the hot state [J. Med. Chem., 1985, pp. 694-698 and J. Med. Chem., 1996, pp. 669-672]. As a preferred solvent, water/alcohol mixtures or dimethylformamide may be mentioned. As a base, triethylamine, N-ethyldiisopropylamine, sodium hydroxide, potassium hydroxide and the like may be used. The temperature is preferably between 40°C and 60°C, and still more preferably the reaction is carried out at 50°C.

c) By peptide coupling between CA-COOH and Rep-R according to conventional techniques known to persons skilled in the art, as described above.

The building block CA-COOH may be obtained in various ways:

- by the action of a building block CA-S-CH_3 on an amino acid or a polyamino acid according to methods known to persons skilled in the art or by any other similar method [J. Am. Chem. Soc., 1956, pp. 1618-1620]. The building block CA-S-CH_3 is obtained in the same manner as above, and the amino or polyamino acid is chosen as a function of the desired compound according to the invention, or
- by the action of an S,S-dimethyltosylimino-thiocarbonimidate or of one of its derivatives on a polyamino acid according to methods known to persons skilled in the art or by any similar method [J. Org. Chem., 1971, pp. 46-48]. Preferably, the procedure is carried out in an ethanolic medium in the presence of a base (for example sodium hydroxide) and at the reflux temperature of the mixture.

By way of example of building blocks CA-COOH which may be obtained by one of the methods described above, the following building blocks may be mentioned:



In all the reactions disclosed above, when the amino substituents present in the various groups may interfere with the reactions carried out, it is preferable to protect them beforehand with compatible radicals which can be introduced and removed without affecting the rest of the molecule. By way of example, the protective radicals may be chosen from the radicals described by T.W. GREENE, *Protective Groups in Organic Synthesis*, J. Wiley-Interscience Publication (1991) or by McOmie, *Protective Groups in Organic Chemistry*, Plenum Press (1973).

Another subject of the invention relates to a composition comprising at least one compound of formula (I) as defined above. In particular, another subject according to the present invention comprises a compound of formula (I) as defined above and a nucleic acid.

When a compound according to the invention and a nucleic acid are brought into contact, they form a complexes by interaction between the positive charges present at physiological pH on the compound according to the invention and the negative charges of the nucleic acid. This complex is called "nucleolipid complex" in the remainder of the text which follows. Preferably, the compound according to the invention and the nucleic acid are present in quantities such that the ratio of the positive charges of the compound to the negative charges of the nucleic acid is between 0.1 and 50, preferably between 0.1 and 20. This ratio can be easily adjusted by persons skilled in the art according to the compound used, the nucleic acid and the desired applications (in particular the type of cells to be transfected).

For the purposes of the invention, "nucleic acid" is understood to mean both a deoxyribonucleic acid and a ribonucleic acid. They may be natural or artificial sequences, and in particular genomic DNA (gDNA), complementary DNA (cDNA), messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), hybrid sequences or synthetic or semisynthetic sequences, oligonucleotides which are modified or otherwise. These nucleic acids may be of human, animal, plant, bacterial or viral origin and the like. They may be obtained by any technique known to persons skilled in the art, and in particular by the screening of libraries, by

chemical synthesis or by mixed methods including the chemical or enzymatic modification of sequences obtained by the screening of libraries. They may be chemically modified.

As regards more particularly deoxyribonucleic acids, they may be single- or double-stranded, as well as short oligonucleotides or longer sequences. In particular, the nucleic acids advantageously consist of plasmids, vectors, episomes, expression cassettes and the like. These deoxyribonucleic acids may carry a replication origin which is functional or otherwise in the target cell, one or more marker genes, sequences for regulating transcription or replication, genes of therapeutic interest, anti-sense sequences which are modified or otherwise, regions for binding to other cellular components, and the like.

Preferably, the nucleic acid comprises an expression cassette consisting of one or more genes of therapeutic interest under the control of one or more promoters and a transcriptional terminator which are active in the target cells.

For the purposes of the invention, "cassette for expression of a gene of interest" is understood to mean a DNA fragment which may be inserted into a vector at specific restriction sites. The DNA fragment comprises a nucleic acid sequence encoding an RNA or a polypeptide of interest and comprises, in addition, the sequences necessary for the expression (enhancer(s),

promoter(s), polyadenylation sequences and the like) of said sequence. The cassette and the restriction sites are designed to ensure insertion of the expression cassette into a reading frame appropriate for transcription and translation.

It is generally a plasmid or an episome carrying one or more genes of therapeutic interest. By way of example, there may be mentioned the plasmids described in patent applications WO 96/26270 and WO 97/10343 which are incorporated into the present application by way of reference.

For the purposes of the invention, gene of therapeutic interest is understood to mean in particular any gene encoding a protein product having a therapeutic effect. The protein product thus encoded may in particular be a protein or a peptide. This protein product may be exogenous, homologous or endogenous in relation to the target cell, that is to say a product which is normally expressed in the target cell when the latter has no pathological condition. In this case, the expression of a protein makes it possible, for example, to palliate an insufficient expression in the cell or the expression of a protein which is inactive or weakly active because of a modification, or to overexpress said protein. The gene of therapeutic interest may also encode a mutant of a cellular protein, having increased stability, a modified activity and the like. The protein product may

also be heterologous in relation to the target cell. In this case, an expressed protein may, for example, supplement or provide an activity which is deficient in the cell, allowing it to combat a pathological condition, or to stimulate an immune response.

Among the therapeutic products for the purposes of the present invention, there may be mentioned more particularly enzymes, blood derivatives, hormones, lymphokines: interleukins, interferons, TNF, and the like (FR 92/03120), growth factors, neurotransmitters or their precursors or synthesis enzymes, trophic factors (BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5, HARP/pleiotrophin and the like), apolipoproteins (ApoAI, ApoAIV, ApoE, and the like, FR 93/05125), dystrophin or a minidystrophin (FR 91/11947), the CFTR protein associated with cystic fibrosis, tumour suppressor genes (p53, Rb, Rap1A, DCC, k-rev, and the like, FR 93/04745), the genes encoding factors involved in coagulation (factors VII, VIII, IX), the genes involved in DNA repair, suicide genes (thymidine kinase, cytosine deaminase), the genes for haemoglobin or other protein carriers, metabolic enzymes, catabolic enzymes and the like.

The nucleic acid of therapeutic interest may also be a gene or an anti-sense sequence, whose expression in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs. Such sequences can, for example, be

transcribed in the target cell into RNAs which are complementary to cellular mRNAs and thus block their translation to protein, according to the technique described in Patent EP 140 308. The therapeutic genes also comprise the sequences encoding ribozymes, which are capable of selectively destroying target RNAs (EP 321 201).

As indicated above, the nucleic acid may also comprise one or more genes encoding an antigenic peptide, which is capable of generating an immune response in humans or in animals. In this specific embodiment, the invention allows either the production of vaccines or the carrying out of immunotherapeutic treatments applied to humans or to animals, in particular against microorganisms, viruses or cancers. They may be in particular antigenic peptides specific for the Epstein-Barr virus, the HIV virus, the hepatitis B virus (EP 185 573), the pseudo-rabies virus, the syncitia forming virus, other viruses, or antigenic peptides specific for tumours (EP 259 212).

Preferably, the nucleic acid also comprises sequences allowing the expression of the gene of therapeutic interest and/or the gene encoding the antigenic peptide in the desired cell or organ. They may be sequences which are naturally responsible for the expression of the gene considered when these sequences are capable of functioning in the infected cell. They may also be sequences of different origin

(responsible for the expression of other proteins, or even synthetic). In particular, they may be promoter sequences of eukaryotic or viral genes. For example, they may be promoter sequences derived from the genome of the cell which it is desired to infect. Likewise, they may be promoter sequences derived from the genome of a virus. In this regard, there may be mentioned, for example, the promoters of the E1A, MLP, CMV and RSV genes, and the like. In addition, these expression sequences may be modified by the addition of activating or regulatory sequences, and the like. The promoter may also be inducible or repressible.

Moreover, the nucleic acid may also comprise, in particular upstream of the gene of therapeutic interest, a signal sequence directing the therapeutic product synthesized in the secretory pathways of the target cell. This signal sequence may be the natural signal sequence of the therapeutic product, but it may also be any other functional signal sequence, or an artificial signal sequence. The nucleic acid may also comprise a signal sequence directing the synthesized therapeutic product toward a particular compartment of the cell.

The compositions according to the invention may, in addition, comprise one or more adjuvants capable of combining with the complexes formed between the compound according to the invention and the nucleic acid, and of improving the transfecting power thereof.

In another embodiment, the present invention therefore relates to compositions comprising a nucleic acid, a compound of formula (I) as defined above and one or more adjuvants capable of combining with the compound (I)/nucleic acid nucleolipid complexes and of improving the transfecting power thereof. The presence of this type of adjuvants (for example lipids, peptides or proteins) can advantageously make it possible to increase the transfecting power of the compounds.

In this regard, the compositions of the invention may comprise, as adjuvant, one or more neutral lipids. Such compositions are particularly advantageous, in particular when the charge ratio R is low. The applicant has indeed shown that the addition of a neutral lipid makes it possible to improve the formation of the nucleolipid particles and to promote the penetration of the particle into the cell by destabilizing its membrane.

More preferably, the neutral lipids used within the framework of the present invention are lipids containing two fatty chains. In a particularly advantageous manner, natural or synthetic lipids which are zwitterionic or lacking ionic charge under physiological conditions are used. They may be chosen more particularly from dioleoylphosphatidylethanolamine (DOPE), oleoylpalmitoylphosphatidylethanolamine (POPE), di-stearoyl, -palmitoyl, -mirystoylphosphatidylethanol-amines as well as their derivatives which are

N-methylated 1 to 3 times, phosphatidylglycerols, diacylglycerols, glycosyldiacylglycerols, cerebrosides (such as in particular galactocerebrosides), sphingolipids (such as in particular sphingomyelins) or asialogangliosides (such as in particular asialoGM1 and GM2).

These different lipids may be obtained either by synthesis or by extraction from organs (for example the brain) or from eggs, by conventional techniques well known to persons skilled in the art. In particular, the extraction of the natural lipids may be carried out by means of organic solvents (see also Lehninger, Biochemistry).

More recently, the applicant has demonstrated that it was also particularly advantageous to use, as adjuvant, a compound involved directly or otherwise in the condensation of the nucleic acid (WO 96/25508). The presence of such a product in a composition according to the invention makes it possible to reduce the quantity of compound of formula (I), with the beneficial consequences resulting therefrom from the toxicological point of view, without any damaging effect on the transfecting activity. Product involved in the condensation of the nucleic acid is intended to define a product which compacts, directly or otherwise, the nucleic acid. More precisely, this product may either act directly at the level of the nucleic acid to be transfected, or may be involved at the level of an

additional product which is directly involved in the condensation of this nucleic acid. Preferably, it acts directly at the level of the nucleic acid. For example, the precompacting agent may be any polycation, for example polylysine. According to a preferred embodiment, this product which is involved in the condensation of the nucleic acid is derived as a whole or in part from a protamine, a histone or a nucleolin and/or from one of their derivatives. Such an agent may also consist, as a whole or in part, of peptide units (KTPKKAKKP) and/or (ATPAKKAA), it being possible for the number of units to vary between 2 and 10. In the structure of the compound according to the invention, these units may be repeated continuously or otherwise. They may thus be separated by linkages of a biochemical nature, for example by one or more amino acids, or of a chemical nature.

Preferably, the compositions of the invention comprise from 0.01 to 20 equivalents of adjuvant(s) for one equivalent of nucleic acids in mol/mol and, more preferably, from 0.5 to 5.

In a particularly advantageous embodiment, the compositions according to the present invention comprise, in addition, a targeting element which makes it possible to orient the transfer of the nucleic acid. This targeting element may be an extracellular targeting element which makes it possible to orient the transfer of DNA toward certain cell types or certain

desired tissues (tumour cells, hepatic cells, haematopoietic cells and the like). It may also be an intracellular targeting element which makes it possible to orient the transfer of the nucleic acid toward certain preferred cellular compartments (mitochondria, nucleus and the like). The targeting element may be linked to the compound according to the invention or also to the nucleic acid as specified above.

Among the targeting elements which may be used within the framework of the invention, there may be mentioned sugars, peptides, proteins, oligonucleotides, lipids, neuromediators, hormones, vitamins or derivatives thereof. Preferably, they are sugars, peptides or proteins such as antibodies or antibody fragments, ligands of cell receptors or fragments thereof, receptors or receptor fragments, and the like. In particular, they may be ligands of growth factor receptors, cytokine receptors, cellular lectin-type receptors, or RGD sequence-containing ligands with an affinity for the receptors for adhesion proteins such as the integrins. There may also be mentioned the receptors for transferrin, HDLs and LDLs, or the folate transporter. The targeting element may also be a sugar which makes it possible to target lectins such as the receptors for asialoglycoproteins or for syalydes such as the sialyde Lewis X, or alternatively an Fab fragment of antibodies, or a single-chain antibody (ScFv).

The combination of the targeting elements with the nucleolipid complexes of the invention may be made by any technique known to persons skilled in the art, for example by coupling to a hydrophobic part or to a part which interacts with the nucleic acid of the compound of general formula (I) according to the invention, or alternatively to a group which interacts with the compound of general formula (I) according to the invention or with the nucleic acid. The interactions in question may be, according to a preferred mode, of an ionic or covalent nature.

According to another variant, the compositions of the invention may also incorporate at least one nonionic surfactant in a sufficient quantity to stabilize the size of the particles of compound of general formula (I)/nucleic acid nucleolipid complexes. The introduction of nonionic surfactants prevents the formation of aggregates, which makes the composition more particularly suitable for an *in vivo* administration. The compositions according to the invention incorporating such surfactants have an advantage from the point of view of safety. They also have an additional advantage in the sense that they reduce the risk of interference with other proteins, given the reduction in the overall charge of the compositions of nucleolipid complexes.

The surfactants advantageously consist of at least one hydrophobic segment, and at least one

hydrophilic segment. Preferably, the hydrophobic segment is chosen from aliphatic chains, polyoxyalkylenes, alkylidene polyesters, polyethylene glycols with a benzyl polyether head and cholesterol, and the hydrophilic segment is advantageously chosen from polyoxyalkylenes, polyvinyl alcohols, polyvinyl pyrrolidones or saccharides. Such nonionic surfactants have been described in application WO 98/34648.

The subject of the invention is also the use of the compounds of general formula (I) as defined above to manufacture a medicament for treating diseases by transfer of nucleic acids (and more generally of polyanions) into primary cells or into established lines. They may be in particular fibroblast cells, muscle cells, nerve cells (neurons, astrocytes, glyal cells), hepatic cells, haematopoietic cell lines (lymphocytes, CD34, dendritic cells and the like), epithelial cells and the like, in differentiated or pluripotent form (precursors).

Such a use is particularly advantageous because the compounds of general formula (I) according to the invention have a reduced cytotoxicity compared with the prior art cationic lipids. The applicant has in particular demonstrated that at very high charge ratios which normally result in the death of the animals following transfection, no apparent cytotoxicity was detected. The compounds of the invention may be used in particular for the *in vitro*,

ex vivo or *in vivo* transfection of nucleic acids. For uses *in vivo*, for example in therapy or for studying the regulation of genes or the creation of animal models of pathological conditions, the compositions according to the invention can be formulated for administration by the topical, cutaneous, oral, rectal, vaginal, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, intratracheal or intraperitoneal route, and the like. Preferably, the compositions of the invention contain a vehicle which is pharmaceutically acceptable for an injectable formulation, in particular for a direct injection into the desired organ, or for administration by the topical route (on the skin and/or the mucous membrane). They may be in particular isotonic sterile solutions, or dry, in particular freeze-dried, compositions which, upon addition, depending on the case, of sterilized water or of physiological saline, allow the constitution of injectable solutions. The nucleic acid doses used for the injection as well as the number of administrations may be adapted according to various parameters, and in particular according to the mode of administration used, the relevant pathological condition, the gene to be expressed, or the desired duration of treatment. As regards more particularly the mode of administration, it may be either a direct injection into the tissues, for example at the level of the tumours, or the circulatory system,

or a treatment of cells in culture followed by their reimplantation *in vivo* by injection or transplantation. The relevant tissues within the framework of the present invention are, for example, the muscles, skin, brain, lungs, liver, spleen, bone marrow, thymus, heart, lymph, blood, bones, cartilages, pancreas, kidneys, bladder, stomach, intestines, testicles, ovaries, rectum, nervous system, eyes, glands, connective tissues, and the like. Advantageously, the transfected tissues are the muscles and the lungs.

The invention relates, in addition, to a method of transferring nucleic acids into cells comprising the following steps:

(1) bringing the nucleic acid into contact with a compound of general formula (I) as defined above, to form a nucleolipid complex, and

(2) bringing the cells into contact with the nucleolipid complex formed in (1).

The cells may be brought into contact with the nucleolipid complex by incubating the cells with said complex (for uses *in vitro* or *ex vivo*), or by injecting the complex into an organism (for uses *in vivo*). The incubation is preferably carried out in the presence, for example, of 0.01 to 1000 µg of nucleic acid per 10^6 cells. For administration *in vivo*, nucleic acid doses of between 0.01 and 10 mg may for example be used.

In the case where the compositions of the invention contain, in addition, one or more adjuvants as defined above, the adjuvant(s) is (are) previously mixed with the compound of general formula (I) according to the invention or with the nucleic acid.

The present invention thus provides a particularly advantageous method for the treatment of diseases by administration of a nucleic acid encoding a protein or which can be transcribed into a nucleic acid capable of correcting said disease, said nucleic acid being combined with a compound of general formula (I) as defined above, under the conditions defined above. More particularly, this method is applicable to diseases resulting from a deficiency in a protein or nucleic product, the administered nucleic acid encoding said protein product or being transcribed into a nucleic product or constituting said nucleic product.

The invention extends to any use of a compound of formula (I) according to the invention for the *in vivo*, *ex vivo* or *in vitro* transfection of cells.

In addition to the preceding arrangements, the present invention also comprises other characteristics and advantages which will emerge from the examples and figures below, which should be considered as illustrating the invention without limiting its scope. In particular, the applicant proposes, with no limitation being implied, various operating protocols as well as reaction intermediates

which can be used to prepare the compounds of general formula (I). Of course, it is within the capability of persons skilled in the art to draw inspiration from these protocols and/or intermediate products in order to develop similar methods to lead to other compounds of general formula (I) according to the invention.

FIGURES

Figure 1: Structure of the synthetic vectors called lipid A, lipid B, lipid c and lipid D in the present invention and which are described in patent application WO 97/18185 incorporated into the present application by way of reference.

Figure 2: Schematic representation of the plasmid pXL2774.

Figure 3: Phase diagram for the compound (1)/DNA nucleolipid complexes. The binding of compound (1) to DNA was determined by following the decrease in the fluorescence (in %, 100% being the fluorescence of the naked DNA) of ethidium bromide (EtBr) (symbol ●, solid line), as described according to the y-axis situated on the right. The size of the particles of complexes (in nm) is indicated on the y-axis situated on the left. The x-axis represents the transfer agents/DNA charge ratio. The size of the nucleolipid complexes without co-lipid is represented by the symbol ■ as a solid line. The size of the nucleolipid complexes containing 25% cholesterol is represented by the symbol

□ as a discontinuous line. The size of the nucleolipid complexes containing 40% DOPE is represented by the symbol ◆ as a discontinuous line. The method does not make it possible to determine the size of the particles above 3 μm.

Figure 4: Activity for *in vitro* gene transfer into HeLa cells of the nucleolipid complexes containing compound (1) according to the present invention without co-lipid (dark-shaded middle bar), with 25% cholesterol (medium-shaded left-hand bar), and with 40 mol % of DOPE (light-shaded right-hand bar), compared with naked DNA. Only the nucleolipid complexes in which the DNA is completely saturated with the compound according to the invention and whose size is between 100 nm and 300 nm were used.

Figure 5: Gene transfer activity *in vitro* into HeLa cells of the nucleolipid complexes formed from compound (3). The expression of luciferase, expressed in pg per well transfected, is represented on the y-axis. The charge ratio between compound (3) and the DNA in nmol/μg is represented on the x-axis. The expression was measured each time for formulations without co-lipid (micelles), with DOPE and with cholesterol.

Figure 6: Gene transfer activity *in vitro* into HeLa cells of the nucleolipid complexes formed from compound (5). The expression of luciferase, expressed in pg per well transfected, is represented on the y-axis. The charge ratio between compound (5) and the DNA in

nmol/µg is represented on the x-axis. The expression was measured each time for formulations without co-lipid (micelles), with DOPE and with cholesterol.

Figure 7: Gene transfer activity *in vitro* into HeLa cells of the nucleolipid complexes formed from compound (6). The expression of luciferase, expressed in pg per well transfected, is represented on the y-axis. The charge ratio between compound (6) and the DNA in nmol/µg is represented on the x-axis. The expression was measured each time for formulations without co-lipid (micelles), with DOPE and with cholesterol.

Figure 8: Gene transfer activity *in vivo* after direct injection into the muscle of the complexes containing compound (1) according to the present invention or the compound of formula $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2COArgN[(CH_2)_{17}CH_3]_2$ (called "lipid A" in the remainder of the text which follows) without co-lipid (dark-shaded bar), with 25% cholesterol (medium-shaded bar), and with 40 mol % of DOPE (light-shaded bar), compared with naked DNA. Only the complexes in which the DNA is completely saturated with lipid and whose size is between 100 nm and 300 nm were used.

Figure 9: The importance of the invention is illustrated by comparing the gene transfer activity of two different lipids, compound (1) according to the invention and lipid A, and of naked DNA via two routes of administration: by the intravenous (iv) route and by

the intramuscular (im) route. Only the complexes in which the DNA is completely saturated with lipid and whose size is between 100 nm and 300 nm were used.

Figure 10: Gene transfer activity *in vivo* 48 hours after i.m. injection of the nucleolipid complexes containing compounds (5) or (6) according to the present invention without co-lipid and at a charge ratio of 0.25/1, compared with naked DNA. The expression is expressed in pg of luciferase per ml. Starting from the left, the bars represent: (a) negative control; (b) naked DNA; (c) compound (5) and (d) compound (6).

MATERIALS AND METHODS

A\ MATERIALS

- The starting amino or polyamino acids (or derivatives thereof) are commercially available. This is the case, for example, for N-(3-aminopropyl)glycine, N-(2-cyanoethyl)glycine or 2,4-diaminobutyric acid, or may be synthesized by conventional methods known to persons skilled in the art.

- The cyclic isothioureas are also commercially available products, such as for example 2-methylthio-2-imidazoline hydriodide, or may be synthesized by conventional methods known to persons skilled in the art.

- The amines substituted with one or more lipid(s) are commercially available or are synthesized from the

corresponding amines and aldehydes by alkylative reduction.

- The products such as triethylamine, trifluoroacetic acid, benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP), dimethylaminopyridine (DMAP), benzyl chloroformate, di-*tert*-butyl dicarbonate are commercially available products. The sodium chloride and sodium carnbonate solutions are saturated. The potassium sulphate solution has a concentration of 0.5 M.

B\ METHODS

1) Physical measurements

The Proton NMR spectra were recorded on Bruker 400 and 600 MHZ spectrometers.

The mass spectra were taken on an API-MS/III.

2) Methods of purification and analysis

a) Direct-phase chromatography conditions

- The thin-layer chromatographies (TLC) were carried out on 0.2 mm thick Merck silica gel plates.

They are developed either under U.V. (254 nm), with ninhydrin, by spraying (light spray) an ethanolic solution of ninhydrin (40 mg/100 cm^3 of ethanol) in order to reveal the amines or the amides by heating to 150°C, with fluorescamine, by spraying a solution (40 mg/100 cm^3 of acetone) in order to reveal the primary amines, with bromocresol green, by spraying a solution (0.1% in 2-propanol) in order to reveal the acids, with vanillin by spraying (light spray) an

ethanolic solution of vanillin (3%) with 3% sulphuric acid followed by heating to 120°C, or with iodine by covering the plate with iodine powder.

- The column chromatographies were carried out on a Merck 60 silica gel having a particle size of 0.063-0.200 mm.

b) Preparative HPLC (High-Performance Liquid Chromatography) purification conditions

The equipment is a set for liquid-phase chromatography in gradient mode, allowing U.V. detection. This preparative chain is composed of the following components:

Pump A: GILSON model 305 equipped with a 50 SC head.

Pump B: GILSON model 303 equipped with a 50 SC head.

Injection loop: 5 ml.

Pressure module: GILSON model 806.

Mixer: GILSON model 811 C equipped with a 23 ml head.

UV detector: GILSON model 119 equipped with a preparative cell.

Fraction collector: GILSON model 202 equipped with No. 21 racks and a 10 ml glass tube.

Integrator: SHIMADZU model C-R6A.

Column: Column C4 (10 mm) made of stainless steel 25 cm long and 2.2 cm in diameter, marketed by VYDAC model 214 TP 1022.

The solution of product to be purified is loaded onto the column by means of the injection loop, the eluent is recovered in fractions of one tube in 30 seconds.

The detector is set at the wavelength of 220 nm and 254 nm.

the mobile phases are defined as follows:

Solvent A		Solvent B	
Demineralized water	2500 cm^3	Acetonitrile for HPLC	2500 cm^3
Trifluoroacetic acid	2 cm^3	Trifluoroacetic acid	2.5 cm^3

Gradient:

Time in minutes	% of solvent A	% of solvent B	Flow rate in cm^3/min
0	90	10	18
10	90	10	18
110	0	100	18
120	0	100	18

c) Analytical chromatography techniques

- The HPLC (High-Performance Liquid Chromatography) analyses were carried out on a Merck-Hitachi apparatus equipped with a HITACHI D 2500 integrator-calculator, an autosampler AS-2000A, an intelligent pump L-6200A, and a vis-UV detector L-4000 with an adjustable wavelength set at 220 nm.

The columns for the analytical separations are Browlee columns made of stainless steel 3 cm long and 0.46 cm in diameter, marketed by APPLIED BIOSYSTEM.

The stationary phase consists of Aquapore Butyl 7 micron. The mobile phases are water (with trifluoroacetic acid) and acetonitrile (with trifluoroacetic acid). The injections are 20 µl of a solution of about 1 mg/cm^3 in a 0.1 cm^3 loop valve. The

flow rate for the analyses is adjusted between 1 cm^3/min and 4 cm^3/min. The pressure is about 180 bars.

The separation conditions are summarized below:

Solvent A		Solvent B	
Demineralized water	2500 cm^3	Acetonitrile for HPLC	2500 cm^3
Trifluoroacetic acid	2 cm^3	Trifluoroacetic acid	2.5 cm^3

Gradient:

Time in minutes	% of solvent A	% of solvent B	Flow rate in cm^3/min
0	60	40	1
3	60	40	1
20	0	100	1
35	0	100	1
35.1	60	40	4
36.1	60	40	4
36.2	60	40	2
44	60	40	2

EXAMPLES

A\ SYNTHESES OF THE COMPOUNDS ACCORDING TO THE INVENTION

Example 1: Synthesis of compound (1) (N-dioctadecylcarbamoylmethyl-2-{3-[4-(2-iminotetrahydropyrimidin-1-yl)butylamino]propylamino}acetamide) from the cationic lipid having the condensed formula $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2COGlyN[(CH_2)_{17}CH_3]_2$ called "lipid B" in the text which follows (whose preparation has been described in patent application WO 98/18185 and whose structure is represented in Figure 1).

0.784 mmol of lipid B is dissolved in 25 cm^3 of methanol in a round-bottomed flask equipped with a magnetic bar, and 10.21 mmol of triethylamine are added. A solution of O-methylisourea and sulphuric acid (1.173 mmol) in water (9 cm^3) is then slowly poured (5 minutes) over the mixture. The mixture is kept at 50°C in an oil bath for twenty hours.

Next, the mixture is concentrated to dryness in a rotary evaporator. The dry extract is solubilized with a solution of water (4 cm^3), and trifluoroacetic acid (1 cm^3). This solution is injected in two portions in preparative HPLC.

The fractions of interest (determined by analytical HPLC) are grouped together, frozen and freeze-dried. 194 mg (0.163 mmol) of salified product are thus obtained.

Yield: 20.8%

$HPLC_{analytical}$: Rt = 15.99 minutes.

^1H NMR spectrum (400 MHz, $(CD_3)_2SO$ d6, δ in ppm): 0.88 (t, J = 6.5 Hz, 6H: CH_3 of the fatty chains); 1.24 (mt, 60H: central CH_2 of the fatty chains); from 1.35 to 1.70 (mt, 4H: 1 CH_2 of each fatty chain); 1.57 (mt, 4H: central $(CH_2)_2$ of the butyl); 1.88 and 1.96 (2 mts, 2H each: central CH_2 of the propyl and central CH_2 of the ring); from 2.85 to 3.35 (2 mts, 16H in total: the 8 NCH_2); 3.81 (broad s, 2H: NCH_2CON); 4.03 (d, J = 5 Hz, 2H: $CONCH_2CON$ of the glycyl); 7.25 and 7.84 (s and broad s respectively, 1H each: the 2 NH of the ring); 8.61

(t, J = 5.5 Hz, 1H: NHCO); 8.70 and 9.02 (2 unres. comp., 1H each: the 2 NH).

MH^+ = 846

Example 2: Synthesis of compound (2) (N-ditetradecyl-carbamoylmethyl-2-{3-[4-(2-iminotetrahydropyrimidin-1-yl)butylamino]propylamino}acetamide) from the compound having the condensed formula $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2COGlyN[(CH_2)_{13}]_2$ called "lipid C" in the text which follows (whose preparation has been described in patent application WO 97/18185 and whose structure is represented in Figure 1).

1.036 mmol of lipid C are dissolved in 30 cm^3 of methanol in a round-bottomed flask equipped with a magnetic bar, and 13.13 mmol of triethylamine are added. A solution of O-methylisourea and sulphuric acid (1.554 mmol) in water (9 cm^3) is then slowly poured (5 minutes) over the mixture. The mixture is kept at 50°C in an oil bath for about twenty hours. The mixture is then concentrated to dryness in a rotary evaporator. The dry extract is solubilized with a solution of water (3 cm^3), ethanol (2 cm^3) and trifluoroacetic acid (0.5 cm^3). This solution is injected in preparative HPLC.

The fractions of interest (determined by analytical HPLC) are grouped together, frozen and freeze-dried. 218 mg (0.2022 mmol) of salified product are finally obtained.

Yield: Y = 19.5%

$HPLC_{analytical}$: Rt = 10.76 minutes.

^1H NMR spectrum (400 MHz, $(CD_3)_2SO$ d6, δ in ppm): 0.88 (t, J = 7 Hz, 6H: CH_3 of the fatty chains); from 1.15 to 1.40 (mt, 44H: central $(CH_2)_{11}$ of the fatty chains); 1.45 and from 1.50 to 1.65 (2 mts, 2H each: 1 CH_2 of each fatty chain); 1.59 (mt, 4H: the 2 central CH_2 of the butyl); 1.91 and 1.97 (2 mts, 2H each: central CH_2 of the propyls); from 2.85 to 3.10 (mt, 10H: the 2 NCH_2 of the butyl - the 2 NCH_2 of one of the 2 propyls - and 1 of the 2 NHC_2 of the other propyl); 3.23 and from 3.30 to 3.50 (2 mts, 5H and 1H respectively: the other NCH_2 of the other propyl and NCH_2 of the fatty chains); 3.79 (unres. comp., 2H: NCH_2CON); 4.03 (d, J = 5 Hz, 2H: $CONCH_2CON$ of the glycyl); 7.27 and from 8.40 to 9.30 (broad s and unres. comp. respectively, 2H and 4H: NH_2^+ CF_3COO^-; NH^+ CF_3COO^- and =NH); 7.88 and 8.61 (s and broad s respectively, 1H each: NHC=N and CONH respectively).

MH^+ = 734

Example 3: Synthesis of compound (3) (2-(3-{4-[3-(4,5-dihydro-1H-imidazol-2-ylamino)propylamino}-N-ditetradecylcarbamoylmethylacetamide) from lipid C (see Example 2 and Figure 1 for its structure).

0.36 mmol of 2-methylmercapto-2-imidazolinium iodide is dissolved in 0.36 cm^3 of 1 N sodium hydroxide in a round-bottomed flask equipped with a bubbler and a magnetic bar. 0.36 mmol of lipid C, previously dissolved in 1.44 cm^3 of 1 N sodium hydroxide, 5 cm^3 of water and 4 cm^3 of ethanol, is added to this solution.

The mixture is kept stirring until the evolution of methyl mercaptan stops (24 hours). The mixture is then concentrated to dryness in a rotary evaporator. The dry extract is solubilized with a solution of water (4 cm^3), ethanol (4 cm^3) and trifluoroacetic acid (0.5 cm^3). This solution is injected in two portions in preparative HPLC.

The fractions of interest (determined by analytical HPLC) are grouped together, frozen and freeze-dried. 213 mg (0.1727 mmol) of salified product are finally obtained.

Yield: Y = 48%

$HPLC_{analytical}$: Rt = 8.90 minutes.

^1H NMR spectrum (400 MHz, $(CD_3)_2SO$ d6 with addition of a few drops of CD_3COOD d4, δ in ppm): 0.87 (t, J = 7 Hz, 6H: CH_3 of the fatty chains); from 1;15 to 1.40 (mt, 44H: central $(CH_2)_{11}$ of the fatty chains); 1.45 and 1.55 (2 mts, 2H each: 1 CH_2 of each fatty chain); 1.65 (mt, 4H: the 2 central CH_2 of the butyl); from 1.80 to 1.95 (mt, 4H: central CH_2 of the propyls); from 2.80 to 3.05 (mt, 10H: the 2 NCH_2 of the butyl - the 2 NCH_2 of one of the 2 propyls - and 1 of the 2 HCH_2 of the other propyl); 3.24 (mt, 6H: the other NCH_2 of the other propyl and NCH_2 of the fatty chains); 3.56 (s, 2H: NCH_2CON); 3.62 (s, 4H: $NCH_2CH_2CH_2N$); 4.02 (d, J = 5 Hz, 2H: $CONCH_2CON$ of the glycyl).

MH^+ = 777

Example 4: Synthesis of compound (4) (2-(3-{bis[3-(4,5-dihydro-1H-imidazol-2-ylamino)propyl]amino}propyl-amino)-N-ditetradecylcarbamoylmethylacetamide) by the method for synthesizing "building blocks".

I) SYNTHESIS OF BOC-GLY-DITETRADECYLAMINE (a)

The group Gly, whose amines are protected with Boc groups (10 mmol), and the ditetradecylamine (10 mmol) are introduced into a 250 ml round-bottomed flask, and 100 cm^3 of dichloromethane are added. The mixture is stirred until complete dissolution is obtained. 30 mmol of N-ethyldiisopropylamine (DIEA) and 11 mmol of benzotriazol-1-yloxytrisdimethylamine phosphonium (BOP) are then added. The pH is kept at 10 by means of the DIEA, and the mixture is stirred for 2 hours. When the reaction is complete, (monitored by CLC and/or HPLC), the dichloromethane is evaporated off and the solid obtained is taken up in ethyl acetate (300 cm^3). The organic phase is washed with a solution of potassium sulphate (4 times 100 cm^3), of sodium carbonate (4 times 100 cm^3), and of sodium chloride (4 times 100 cm^3). The organic phase is then dried over magnesium sulphate, filtered and evaporated under vacuum. The product (a) is obtained with a yield of 93%.

TLC: R_f = 0.9 ($CHCl_3$/MeOH, 9:1)

MH^+ = 567

II) SYNTHESIS OF $[Z\text{-}NH(CH_2)_3]_2\text{-}N\text{-}(CH_2)_3\text{-}NH\text{-}Boc\text{-}CH_2\text{-}COOH$ (b)

1) Synthesis of $NC\text{-}(CH_2)_2\text{-}NH\text{-}Boc\text{-}CH_2\text{-}COOH$ (c)

The amine of N-(cyanoethyl)glycine (0.1 mol/amine, commercial) is solubilized in 1 N sodium hydroxide ($200\ cm^3$/amine) and dioxane ($200\ cm^3$). The solution is stirred on an ice bath and then a solution of O-(*t*-butoxucarbonyl)$_2$ or of p-chlorobenzyloxycarbonyl (ClZ, 0.14 mol/amine) in $200\ cm^3$ of dioxane is added dropwise. The pH is kept at a value greater than 9. The mixture is then stirred at about 20°C overnight. The dioxane is evaporated under vacuum and then the mixture is acidified to pH 3 with the aid of a potassium sulphate solution. The insoluble matter is extracted with ethyl acetate (3 times $100\ cm^3$) and then washed with a sodium chloride solution (2 times $100\ cm^3$). The organic phase is dried over magnesium sulphate, filtered and evaporated under vacuum. The product (c) of formula $NC\text{-}(CH_2)_2\text{-}NH\text{-}Boc\text{-}CH_2COOH$ is obtained with a yield of 98%.

TLC: R_f = 0.66 ($CHCl_3$/MeOH, 8:2)

MH^+ = 229

2) Synthesis of $NH_2\text{-}(CH_2)_3\text{-}NH\text{-}Boc\text{-}CH_2\text{-}COOH$ (d)

50 mmol of product (c) of formula $NC\text{-}(CH_2)_2\text{-}NH\text{-}Boc\text{-}CH_2\text{-}COOH$ are introduced into a 1 litre stainless steel autoclave. A solution of $10\ cm^3$ of ethanol (95%) and of 3.3 g of sodium hydroxide (80 mol) is prepared at the same time in a beaker. When the

sodium hydroxide has dissolved, 2 cm^3 of raney Nickel on carbon are introduced. The autoclave is closed. The initial hydrogenation pressure is about 52 bar, and it decreases to about 48.5 bar overnight at room temperature (20°C). The suspension is filtered on paper, the filter is washed with ethanol (4 times 25 cm^3), and the filtrates are concentrated to dryness under vacuum. The product (d) is obtained which is used without further purification in the next stage.

TLC: R_f = 0.12 ($CHCl_3$/MeOH, 6:4)

MH^+ = 233

3) Synthesis of $[NC(CH_2)_2]_2$-N-$(CH_2)_3$-NH-Boc-CH_2-COOH (e)

The product (d) of formula NH_2-$(CH_2)_3$-NH-Boc-CH_2-COOH (0.05 mol) and sodium hydroxide (0.1 mol) are solubilized in 150 cm^3 of water, in a round-bottomed flask. The solution is cooled on an ice bath. Acrylonitrile (0.12 mol) is slowly poured in, with vigorous stirring, while the temperature of the mass is kept below 20°C. The reaction mixture is kept overnight at room temperature (20°C). The mixture is then kept at 50°C for 2 hours. The solvent is evaporated under vacuum and then the mixture is acidified to pH 3 with a solution of potassium sulphate. The insoluble matter is extracted with ethyl acetate (3 times 200 cm^3), and then washed with a sodium chloride solution (2 times 100 cm^3). The organic phase is

dried over magnesium sulphate and then filtered and evaporated under vacuum. The "crude material" is optionally purified on a silica column. The product (e) is obtained with a yield of 50%.

TLC: R_f = 0.75 ($CHCl_3$/MeOH, 6:4)

MH^+ = 339

4) Synthesis of $[Z\text{-}NH(CH_2)_3]_2\text{-}N\text{-}(CH_2)_3\text{-}NH\text{-}Boc\text{-}CH_2\text{-}COOH$ (b)

The product (e) of formula $[NC(CH_2)_2]_2\text{-}N\text{-}(CH_2)_3\text{-}NH\text{-}Boc\text{-}CH_2\text{-}COOH$ (50 mmol) is introduced into a 1 litre stainless steel autoclave. A solution of 10 cm^3 of ethanol (95%) and of 3.3 g of sodium hydroxide (80 mol) is prepared at the same time in a beaker. When the sodium hydroxide has dissolved, this solution is introduced into the autoclave. A nitrogen stream is passed through the autoclave and 2 cm^3 of Raney Nickel on carbon are introduced. The autoclave is closed. The initial hydrogenation pressure is about 52 bar, and it decreases to about 48.5 bar overnight at room temperature (20°C). The suspension is filtered on paper, the filter is washed with ethanol (4 times 25 cm^3), and the filtrates are concentrated to dryness under vacuum. A white solid is obtained which is used without further purification after TLC analysis.

TLC: R_f = 0.14 ($CHCl_3$/MeOH, 6:4)

The solid obtained above is solubilized in 1 N sodium hydroxide (200 cm^3/amine) and dioxane (200 cm^3). The solution is stirred on an ice bath

and then a solution of (t-butoxycarbonyl)$_2$O or of p-chlorobenzyloxycarbonyl (0.14 mol/amine) in 200 cm^3 of dioxane is then added dropwise. The pH is kept at a value greater than 9. The mixture is then stirred at room temperature (20°C) overnight. The dioxane is evaporated under vacuum and then the mixture is acidified to pH 3 with the aid of a potassium sulphate solution. The insoluble matter is extracted with ethyl acetate (3 times 100 cm^3) and then washed with a sodium chloride solution (2 times 100 cm^3). The organic phase is dried over magnesium sulphate, filtered and evaporated under vacuum. The products are analysed by TLC and/or HPLC.

The crude product is purified on a silica column (dichloromethane/methanol, 8:2).

The product (b) is obtained with a yield of 66% relative to the product (d).

TLC: R_f = 0.42 ($CHCl_3$/MeOH, 6:4)

MH^+ = 615

III) SYNTHESIS OF [Z-NH$(CH_2)_3$]$_2$-N-$(CH_2)_3$-NH-Boc-CH_2-COGlyN[$(CH_2)_{13}$-CH_3]$_2$ (f)

Product (a) whose amines are protected with Boc groups (1 mmol) is introduced into a round-bottomed flask equipped with a magnetic bar. 30 cm^3 of trifluoroacetic acid at 4°C are added and then the solution is stirred for one hour. When the reaction is complete (monitored by TLC and/or HPLC), the trifluoroacetic acid is

evaporated under vacuum and then the product is dried by coevaporation with 3 times 30 cm^3 of ethyl ether.

HPLC: R_t = 12.86 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100].

The produced obtained (Gly-ditetradecylamine, 10 mmol) and the product (b) (10 mmol) are introduced into a 250 cm^3 round-bottomed flask, dichloromethane (100 cm^3) is added and the mixture is stirred until complete dissolution is obtained. 30 mmol of N-ethyldiisopropyl-amine (DIEA) and 11 mmol of BOP hexafluorophosphate are then added. The pH is kept at 10 by means of DIEA and the mixture is stirred for two hours. When the reaction is complete (monitored by TLC and/or HPLC), the dichloromethane is evaporated and the solid obtained is taken up in ethyl acetate (300 cm^3). The organic phase is washed with a potassium sulphate solution (4 times 100 cm^3), of sodium carbonate (4 times 100 cm^3), and of sodium chloride (4 times 100 cm^3). The organic phase is dried over magnesium sulphate, filtered and evaporated under vacuum. The products are used without further purification. The product (f) is obtained with a yield of 75% after purification on a silica column (dichloromethane/methanol, 8:2).

TLC: R_f = 0.86 ($CHCl_3$/MeOH, 8:2)

HPLC: R_t = 17.44 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

IV) SYNTHESIS OF $[NH_2(CH_2)_3]_2$-N-$(CH_2)_3$-NH-Boc-CH_2-COGlyN$[(CH_2)_{13}$-$CH_3]_2$ (g)

Product (f), whose amines are protected, is introduced into a round-bottomed flask equipped with a magnetic bar and dissolved in 10 cm^3 of methanol per gram of product. Palladium on carbon (10%, 1 g/g of product) and ammonium formate (1 g/g of product) are added at room temperature. Hydrogenolysis is monitored by HPLC. After two hours, the reaction is complete, the mixture is filtered and the filter washed with three times 10 cm^3 of methanol per gram of product. Double-distilled water is added and the solution is frozen and freeze-dried, or the filtrate is concentrated to dryness and the solid is taken up in ethyl acetate (300 cm^3). The organic phase is washed with a sodium carbonate solution (twice 100 cm^3), and a sodium chloride solution (twice 100 cm^3), and then it is dried over magnesium sulphate, filtered and evaporated under vacuum. The products are analysed by HPLC and are used without further purification. The product (g) is obtained with a yield of 40% relative to the product (f).

HPLC: R_t = 9.62 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

MH^+:: 795

V) SYNTHESIS OF COMPOUND (4)

Product (g), which contains the primary amine to be modified (1 mmol/amine) is solubilized in dichloromethane (10 cm^3) and then 2-methylthio-

imidazoline hydriodide (1.2 mmol/amine) and triethylamine (3 mmol/amine) are added. The mixture is stirred at room temperature (20°C) until the evolution of methyl sulphide stops. At the end of the reaction (monitored by HPLC), the dichloromethane is evaporated under vacuum.

The product obtained, whose amines are protected by Boc groups (1 mmol) is introduced into a round-bottomed flask equipped with a magnetic bar. 30 cm^3 of trifluoroacetic acid at 4°C are added and then the solution is stirred for one hour. When the reaction is complete (monitored by TLC and/or HPLC), the trifluoroacetic acid is evaporated under vacuum and then the product is dried by coevaporation with 3 times 30 cm^3 of ethyl ether.

The product obtained is purified by preparative HPLC and the fractions analysed by HPLC. The compound (4) according to the present invention is thus obtained with a yield of 34%.

HPLC: R_t = 10.07 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

^1H NMR spectrum (400 MHz, $(CD_3)_2SO$ d_6 at a temperature of 383 K, d in ppm): 0.92 (t, J = 7 Hz, 6H: CH_3 of the fatty chains); from 1.25 to 1.45 (mt, 44H: (central $(CH_2)_{11}$ of the fatty chains); 1.57 (mt, 4H : 1 CH_2 of each fatty chain); from 1.70 to 1.90 (mt, 6H : central CH_2 of the propyls); from 2.50 to 3.40 (mt, 16H : the 2 NHC_2 of the propyls and the NCH_2 of the fatty chains);

3.68 (s, 8H : the 2 NCH_2CH_2N); 3.72 (broad s, 2H : NCH_2CON); 4.06 (s, 2H : $CONCH_2CON$ of the glycyl).

MH^+: 831

Example 5: Synthesis of compound (5):

(N-Ditetradecylcarbamoylmethyl-2-{3-[3-(1,4,5,6-tetrahydropyrimidin-2-ylamino)propylamino]propylamino}-acetamide) by the method of synthesis of "building blocks".

I) SYNTHESIS OF BOC-GLY-DITETRADECYLAMINE (a)

The procedure is carried out in the same manner as in the preceding example. Product (a) is obtained with a yield of 93%.

TLC: R_f = 0.9 ($CHCl_3$/MeOH, 9:1)

MH^+: 567

II) SYNTHESIS OF $Z\text{-}NH(CH_2)_3\text{-}N\text{-}Boc\text{-}(CH_2)_3\text{-}N\text{-}Boc\text{-}CH_2\text{-}COOH$ (b)

1) Synthesis of $NC\text{-}(CH_2)_2\text{-}NH\text{-}Boc\text{-}CH_2\text{-}COOH$ (c)

The procedure is carried out in the same manner as above in Example 4. Product (c) is obtained with a yield of 98%.

TLC: Rf = 0.66 ($CHCl_3$/MeOH, 8:2)

MH^+: 229

2) Synthesis of $NH_2\text{-}(CH_2)_3\text{-}NH\text{-}Boc\text{-}CH_2\text{-}COOH$ (d)

Product (d) is obtained in the same manner as above in Example 4.

TLC: R_f = 0.12 ($CHCl_3$/MeOH, 6:4)

MH^+: 233

3) Synthesis of $NC(CH_2)_2$-N-Boc-$(CH_2)_3$-NH-Boc-CH_2-COOH (e)

Product (d) (0.05 mol) and sodium hydroxide (0.1 mol) are solubilized in 150 cm^3 of water in a round-bottomed flask. The solution is cooled on an ice bath. Acrylonitrile (0.05 mol) is slowly poured in, with vigorous stirring, while the temperature of the mass is kept below 20°C. The reaction mixture is kept overnight at room temperature (20°C).

The solvent is evaporated under vacuum and then the mixture is acidified to pH 3 with a potassium sulphate solution. The insoluble matter is extracted with ethyl acetate (3 times 200 cm^3), and then washed with a sodium chloride solution (twice 100 cm^3). The organic phase is dried over magnesium sulphate and then filtered and evaporated under vacuum. The product obtained is optionally purified on a silica column.

The product obtained (0.1 mol/amine) is solubilized in 1 N sodium hydroxide (200 cm^3/amine) and dioxane (200 cm^3). The solution is stirred on an ice bath and then a solution of $(Boc)_2O$ or of p-chlorobenzyloxycarbonyl (0.14 mol/amine) in 200 cm^3 of dioxane is added dropwise. The pH is kept at a value greater than 9. The mixture is then stirred at room temperature (20°C) overnight. The dioxane is evaporated under vacuum and then

the mixture is acidified to pH 3 with the aid of a potassium sulphate solution. The insoluble matter is extracted with ethyl acetate (3 times 100 cm^3) and then washed with a sodium chloride solution (twice 100 cm^3). The organic phase is dried over magnesium sulphate, filtered and evaporated under vacuum. The products are analysed by TLC and/or HPLC.

Product (e) is thus obtained with a yield of 93%.

TLC: R_f = 0.75 ($CHCl_3$/MeOH, 8:2)

MH^+: 386

4) Synthesis of Z-NH-$(CH_2)_3$-N-Boc-$(CH_2)_3$-N-Boc-CH_2-COOH (b)

Product (e) (50 mmol) is introduced into a one-litre stainless steel autoclave. A solution of 10 cm^3 of ethanol (95%) and of 3.3 g of sodium hydroxide (80 mol) is prepared at the same time in a beaker. When the sodium hydroxide has dissolved, this solution is introduced into the autoclave. A nitrogen stream is passed through the autoclave and 2 cm^3 of Raney Nickel on carbon are introduced. The autoclave is closed. The initial hydrogenation pressure is about 52 bar and it decreases to about 48.5 bar overnight at room temperature (20°C). The suspension is filtered on paper, the filter is washed with ethanol (4 times 25 cm^3) and the filtrates are concentrated to dryness under

vacuum. A white solid is obtained which is used without further purification after TLC analysis.

TLC: R_f = 0.14 ($CHCl_3$/MeOH, 6:4)

The product obtained (0.1 mol/amine) is solubilized in 1 N sodium hydroxide (200 cm^3/amine) and dioxane (200 cm^3). The solution is stirred on an ice bath and then a p-chlorobenzyloxycarbonyl solution (0.14 mol/amine) in 200 cm^3 of dioxane is added dropwise. The pH is kept at a value greater than 9. The mixture is then stirred at room temperature (20°C) overnight. The dioxane is evaporated under vacuum and then the mixture is acidified to pH 3 with the aid of a potassium sulphate solution. The insoluble matter is extracted with ethyl acetate (3 times 100 cm^3) and then washed with a sodium chloride solution (twice 100 cm^3). The organic phase is dried over magnesium sulphate, filtered and evaporated under vacuum. The product obtained is purified on a silica column (dichloromethane/methanol, 9:1). The products are analysed by TLC and/or HPLC. Product (b) is obtained with a yield of 32% relative to product (d).

TLC: R_f = 0.63 ($CHCl_3$/MeOH, 9:1)

MH^+: 523

III) SYNTHESIS OF 2-methylsulphanyl-1,4,5,6-tetrahydro-pyridmidine (f)

3,4,5,6-Tetrahydro-2-pyrimidinethiol (0.0103 mol) is loaded into a round-bottomed flask, with stirring and under a nitrogen stream, and 5 cm^3 of methanol and 0.65 cm^3 of methyl iodide (0.0105 mol) are added. The mixture is heated under reflux for 1 hour and is then allowed to cool to room temperature (20°C). The product is precipitated by addition of 5 cm^3 of ethyl ether. The precipitate is filtered and then washed with ethyl ether. The product is then dried overnight at a pressure of 34 mbar.

1.5 g (0.0041 mol) of product (VI) are obtained, that is to say a yield of 40%.

TLC: R_f = 0.25 ($CHCl_3$/MeOH, 9:1)

MH^+: 131

d) SYNTHESIS OF Z-NH$(CH_2)_3$-N-Boc$(CH_2)_3$-N-Boc-CH_2-COGlyN$[CH_2)_{13}$-$CH_3]_2$ (g)

Product (a), whose amines are protected with Boc groups (1 mmol) is introduced into a round-bottomed flask equipped with a magnetic bar. 30 cm^3 of trifluoroacetic acid at 4°C are added and then the solution is stirred for one hour. When the reaction is complete (monitored by TLC and/or HPLC), the trifluoroacetic acid is evaporated under vacuum and then the product obtained is dried by coevaporation with 3 times 30 cm^3 of ethyl ether.

HPLC: R_t = 12.86 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

The product obtained (10 mmol) and the product (b) (10 mmol) are introduced into a 250 cm^3 round-bottomed flask. Dichloromethane (100 cm^3) is added and the mixture is stirred until complete dissolution is obtained. 30 mmol of DIEA and 11 mmol of BOP are then added. The pH is kept at 10 by means of the DIEA and the mixture is stirred for two hours. When the reaction is complete (monitored by TLC and/or HPLC), the dichloromethane is evaporated and the solid obtained is taken up in ethyl acetate (300 cm^3). The organic phase is washed with a solution of potassium sulphate (4 times 100 cm^3), of sodium carbonate (4 times 100 cm^3), and of sodium chloride (4 times 100 cm^3). The organic phase is dried over magnesium sulphate, filtered and evaporated under vacuum. The products are used without further purification.

After purification on a silica column (dichloromethane/methanol, 8:2), product (g) is obtained with a yield of 85%).

TLC: R_f = 0.9 ($CHCl_3$/MeOH, 9:1)

HPLC: R_t = 19.79 in, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

V) SYNTHESIS OF $NH_2(CH_2)_3]_2$-N-Boc-$(CH_2)_3$-NH-Boc-CH_2-COGlyN$[(CH_2)_{13}$-$CH_3]_2$ (h)

Product (g) is introduced into a round-bottomed flask equipped with a magnetic bar and dissolved in 10 cm^3 of

methanol/g of product. Palladium on carbon (10%, 1 g/g of product) and ammonium formate (1 g/g of product) are added at room temperature (20°C). The hydrogenolysis is monitored by HPLC. After two hours, the reaction is complete, the mixture is filtered and the filter is washed with 3 times 10 cm^3 of methanol/g of product. Double-distilled water is added and the solution is frozen and freeze-dried, or the filtrate is concentrated to dryness and the solid is taken up in ethyl acetate (300 cm^3). The organic phase is washed with a solution of sodium carbonate (twice 100 cm^3), and a solution of sodium chloride (twice 100 cm^3) and then it is dried over magnesium sulphate, filtered and evaporated under vacuum. The products are analysed by HPLC and are used without further purification. Product (h) is obtained with a yield of 93% relative to product (g).

TLC: R_f = 0.42 ($CHCl_3$/MeOH, 6:4)

HPLC: R_t = 14.66 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

MH^+: 838

VI) SYNTHESIS OF COMPOUND (5)

Product (h) containing the primary amine to be modified (1 mmol/amine) is solubilized in dichloromethane (10 cm^3) and then product (f) (1.2 mmol/amine) and triethylamine (1.3 mmol/amine) are added. The mixture is stirred at room temperature (20°C) until the evolution of methyl sulphide stops. At the end of the

reaction (monitored by HPLC), the dichloromethane is evaporated under vacuum.

The product obtained is purified by preparative HPLC and the fractions analysed by HPLC. Compound (5) is thus obtained with a yield of 38%.

HPLC: R_t = 8.42 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

^1H NMR spectrum (400 MHz, $(CD_3)_2SO$ d_6 δ in ppm): 0.86 (t, J = 7 Hz, 6H : CH_3 of the fatty chains); from 1.10 to 1.35 (mt, 44H : central $(CH_2)_{11}$ of the fatty chains); 1.44 and 1.53 (2 mts, 2H each : 1 CH_2 of each fatty chain); from 1.80 to 2.00 (mt, 6H : central CH_2 of the propyls and CH_2 of 1,4,5,6-tetrahydropyrimidine); from 2.80 to 3.10 (mt, 10H : NCH_2 of the propyls and NCH_2 of 1,4,5,6-tetrahydropyrimidine); from 3.14 to 3.45 (mt : the 6H corresponding to the $=NCH_2$ of 1,4,5,6-tetrahydropyrimidine and to the $=NCH_2$ of the fatty chains); 3.81 (unres. comp., 2H : NCH_2CON); 4.04 (d, J = 5 Hz, 2H: $CONCH_2CON$ of the glycyl); 7.89 = 8.62 - 8.75 and 9.01 (4 unres. comp., 8H in total : the exchangeables and OH of the CF_3COOH).

MH^+: 720

Example 6: Synthesis of compound (6): N-Dioctadecyl-carbamoylmethyl-2-{3-[3-(1,4,5,6-tetrahydropyrimidin-2-ylamino)propylamino]propylamino}acetamide) by the method of synthesis of "building blocks".

I) SYNTHESIS OF BOC-GLY-DITETRADECYLAMINE (a)

The procedure is carried out in the same manner as in the preceding example. Product (a) is obtained with a yield of 93%.

TLC: R_f = 0.9 ($CHCl_3$/MeOH, 9:1)

MH^+: 567

II) SYNTHESIS OF Z-$NH(CH_2)_3$-N-Boc-$(CH_2)_3$-N-Boc-CH_2-COOH (b)

1) Synthesis of NC-$(CH_2)_2$-NH-Boc-CH_2-COOH (c)

The procedure is carried out in the same manner as above in Example 5. Product (c) is obtained with a yield of 98%.

TLC: Rf = 0.66 ($CHCl_3$/MeOH, 8:2)

MH^+: 229

2) Synthesis of NH_2-$(CH_2)_3$-NH-Boc-CH_2-COOH (d)

Product (d) is obtained in the same manner as above in Example 5.

TLC: R_f = 0.12 ($CHCl_3$/MeOH, 6:4)

MH^+: 233

3) Synthesis of NC$(CH_2)_2$-N-Boc-$(CH_2)_3$-NH-Boc-CH_2-COOH (e)

The procedure is carried out in the same manner as above in Example 5. Product (e) is thus obtained with a yield of 93%.

TLC: R_f = 0.75 ($CHCl_3$/MeOH, 8:2)

MH^+: 386

4) Synthesis of Z-NH-$(CH_2)_3$-N-Boc-$(CH_2)_3$-N-Boc-CH_2-COOH (b)

The procedure is carried out in the same manner as above in Example 5. A white solid is obtained which is used without further purification after a TLC analysis.

TLC: R_f = 0.14 ($CHCl_3$/MeOH, 6:4)

The product obtained is used in the same manner as above so as to protect the terminal amine with a benzyloxycarbonyl group. Product (b) is thus obtained with a yield of 32% relative to product (d).

TLC: R_f = 0.63 ($CHCl_3$/MeOH, 9:1)

MH^+: 523

III) SYNTHESIS OF 2-methylsulphanyl-1,4,5,6-tetrahydro-pyridmidine (f)

The procedure is carried out in the same manner as above in Example 5. 1.5 g (0.0041 mol) of product (f) are thus obtained, that is to say a yield of 40%.

TLC: R_f = 0.25 ($CHCl_3$/MeOH, 9:1)

MH^+: 131

IV) SYNTHESIS OF Z-NH$(CH_2)_3$-N-Boc$(CH_2)_3$-N-Boc-CH_2-COGlyN$[(CH_2)_{17}$-$CH_3]_2$ (g)

The procedure is carried out in the same manner as above in Example 5. Product (a) is thus obtained whose Boc groups have been cleaved.

HPLC: R_t = 19.44 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

This product obtained is used in the same manner with product (b) as above in Example 5. After purification on a silica column (dichloromethane/methanol, 8:2), product (g) is obtained with a yield of 84%.

TLC: R_f = 0.9 ($CHCl_3$/MeOH, 9:1)

HPLC: R_t = 23.95 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

V) SYNTHESIS OF $NH_2(CH_2)_3]_2$-N-Boc-$(CH_2)_3$-NH-Boc-CH_2-COGlyN$[(CH_2)_{17}$-$CH_3]_2$ (h)

The procedure is carried out in the same manner as above with Example 5. Product (h) is obtained with a yields of 73% relative to product (g).

TLC: R_f = 0.28 ($CHCl_3$/MeOH, 6:4)

HPLC: R_t = 20.59 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

MH^+: 838

VI) SYNTHESIS OF COMPOUND (6)

The procedure is carried out in the same manner as above in Example 5. Compound (6) is thus obtained with a yield of 68%.

HPLC: R_t = 15.83 min, (H_2O/MeCN: 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

^{1}H NMR spectrum (500 MHz, $(CD_3)_2SO$ d_6 δ in ppm): 0.88 (t, J = 7 Hz, 6H : CH_3 of the fatty chains); from 1.15 to 1.35 (mt, 60H : central $(CH_2)_{15}$ of the fatty chains); 1.46 and 1.54 (2 mts, 2H each : 1 CH_2 of each fatty chain); from 1.80 to 2.00 (mt, 6H : central CH_2 of the propyls and CH_2 of 1,4,5,6-tetrahydropyrimidine); from

2.85 to 3.05 (mt, 10H : NCH_2 of the propyls and NCH_2 of 1,4,5,6-tetrahydropyrimidine); from 3.15 to 3.45 (mt : the 6H corresponding to the $=NCH_2$ of 1,4,5,6-tetrahydropyrimidine and to the NCH_2 of the fatty chains); 3.81 (unres. comp., 2H : NCH_2CON); 4.04 (d, J = 5 Hz, 2H = $CONCH_2CON$ of the glycyl); 7.88 = 8.61 - 8.74 and 8.99 (4 unres. comp., 8H in total : the exchangeables and OH of the CF_3COOH).

MH^+: 832

B\USE OF THE TRANSFECTION AGENTS ACCORDING TO THE INVENTION

Example 7: preparation of nucleolipid complexes

This example illustrates the preparation of nucleolipid complexes according to the invention.

The compound used in this example is compound (1) in solution in chloroform. 10 nmol quantities of compound (1) (that is to say 11.8 μg) per μg of DNA were used. In some cases, a neutral co-lipid, Cholesterol or DOPE, is previously mixed with the compound. A fine lipid film forms when the chloroform is evaporated with the aid of a slight stream of argon, and then it is rehydrated in a mixture of 5% dextrose and 10 mM sodium chloride overnight at 4°C. The samples are then treated with ultrasound for 5 minutes, heated at 65°C for 30 minutes and finally treated again with ultrasound for 5 minutes. Lipid suspensions are thus obtained which are stored at 4°C until they are used.

The DNA used is the plasmid pXL2774 (Figure 2) in solution in a mixture of 5% dextrose and 20 mM sodium chloride at a concentration of 0.5 mg/ml or 1.0 mg/ml. The plasmid pXL2774 has the following characteristics:

- level of endotoxins less than 50 EU/mg,
- level of supercoiled DNA greater than 60%,
- content of RNA, that is to say of mRNA, tRNA and ribosomal RNA (determined by HPLC) less than 5%,
- level of chromosomal DNA less than 1%,
- protein content less than 1%,
- osmolarity less than 15 mosmol/kg.

The nucleolipid complexes according to the invention are prepared by rapidly mixing equal volumes of DNA solution and lipid suspension as described above. The quantity of compound complexed with the DNA varies from 0.5 nmol/μg of DNA to 12 nmol/μg of DNA.

Example 8: behaviour of the complexes formed at different charge ratios

This example illustrates the behaviour of the nucleolipid complexes according to the invention at different charge ratios. The impact of the addition of a neutral co-lipid is also illustrated.

The size of the complexes was first of all analysed by measuring the hydrodynamic diameter by dynamic light scattering (Dynamic Laser Light Scattering) with the aid of a Coulter N4plus apparatus. The samples are diluted 20-fold in a solution

containing 5% dextrose and 20 mM sodium chloride in order to avoid multiple diffusions. The effect of the cycloamidine group, of the lipid composition and of the charge ratio on the size of the nucleolipid complexes according to the invention was thus studied.

Three possible phases can be distinguished when the charge ratio between compound (1) according to the invention and the DNA is increased. These three phases determine the therapeutic potential of compound (1). Figure 3 illustrates these 3 phases for compound (1). The same behaviour can be observed for other compounds according to the invention.

At a low charge ratio, the DNA is not saturated with compound (1). Naked DNA still remains, and the complexes are negatively charged overall. The particles are small in size (between 100 and 300 nm). This phase is called "Phase A".

The fact that the DNA is not completely saturated with compound (1) means that the DNA is not completely protected by it. The DNA can therefore be subjected to degradation by enzymes (DNAses). Moreover, since the complexes are negative overall, the crossing of the cell membrane is difficult. For these reasons, the nucleolipid complexes of phase A are of a much lower efficiency in transfection.

At an intermediate charge ratio, the DNA is completely saturated with compound (1), and the complexes are neutral or slightly positive overall.

This phase is unstable because the ionic repulsions are minimal and a "crosslinking" phenomenon may occur. The size of the particles is well above the limit of detection by dynamic light scattering (much greater than 3 μm). This unstable phase is called "phase B". Such a size of complexes is not suited to uses by injection. However, this does not necessarily mean that the complexes are inactive in phase B, but they are only in a formulation which is not appropriate for their injection for pharmaceutical purposes.

At a relatively high charge ratio, the DNA is oversaturated with compound (1), and the complexes are positive overall. Because of the strong repulsions between the positive charges, this phase is stable. It is designated by the name "phase C". Unlike phase A, the nucleolipid complexes are in a form such that the DNA is very well protected against enzymes, and their overall positive charge facilitates the crossing of the cell membrane of anionic nature. The phase C complexes are therefore particularly suited to use for the transfer of nucleic acids into cells.

In addition to the cycloamidine group of the compound according to the invention, the use of a neutral co-lipid has a strong impact on the stability of the complexes, as is illustrated in Figure 3. The co-lipids added are either DOPE (cationic lipid/DOPE = 3/2), or cholesterol (cationic lipid/cholesterol = 3/1). In general, the addition of the neutral co-lipid

increases the instability of the complexes, which causes an increase in the quantity of compound required to obtain phase C. This is very clearly illustrated in Figure 3 when the charge ratio at which phase C is obtained in the presence and in the absence of co-lipid is compared.

It should be noted that the values of the charge ratio which delimit the three phases A, B and C depend on the compound used. Thus, these values can vary very widely from one compound to another.

Finally, the affinity of the compound for the DNA as a function of the charge ratio was studied. For that, the reduction in fluorescence after the addition of 3 μg of ethidium bromide (EtBr) was measured. Indeed, the replacement of the ethidium bromide of the DNA by the compound is an indication of binding to the DNA.

The formulation used is diluted 20-fold to a final concentration of 25 μg of DNA/ml. The relative fluorescence measured for naked DNA is defined as being 100%. The level of binding with compound (1) is represented by the reduction in the relative fluorescence of the sample. Figure 3 shows that the fluorescence decreases when the charge ratio increases, which means that a greater quantity of compound (1) is available to bind to the DNA (the more the fluorescence decreases, the more a large quantity of compound binds to the DNA until saturation is reached).

In this manner, it has been shown that the affinity of compound (1) according to the invention for the DNA is determined by the cycloamidine group, but not by the addition of a co-lipid.

Example 9: transfection *in vitro* with compound (1)

This example illustrates the capacity of compound (1) according to the invention to transfect DNA into cells *in vitro*, compared with nonformulated DNA.

24-well microplates are inoculated with 60,000 HeLa cells (ATCC) per well, and transfected 24 hours later. Complexes containing 1 μg of DNA are diluted in 0.5 ml of DMEM culture medium (Gibco/BRL) in the absence of serum, and then added to each well. The cells are incubated at 37°C for 4 hours. The medium containing the complexes is then removed and replaced with a mixture of DMEM and 10% foetal calf serum. Next, the cells are again cultured for 24 hours. Finally, the cells are lysed and tested using a luciferase test kit (Promega) and a Dynex MLX luminometer.

The results indicated in Figure 4 underline the difference between the performance of the naked DNA compared with the compound (1)/DNA complexes of the invention which are completely saturated: no luciferase activity could be detected (sensitivity of the apparatus less than 1 pg per well) after transfection *in vitro* of naked DNA, whereas the gene transfer

activity of the complexes according to the invention varies from 200 pg/well to 8000 pg/well.

This example therefore clearly shows the advantageous use of compound (1) according to the invention for the transfection of cells *in vitro*.

Example 10: transfection *in vitro* with compounds (3), (5) and (6)

This example illustrates the capacity of compounds (3), (5) and (6) according to the invention to transfect DNA into cells *in vitro*, compared with nonformulated DNA.

The transfection is carried out according to the preceding protocol of Example 9, into HeLa cells. The results are illustrated in Figures 5, 6 and 7. It is thus observed that these 3 compounds have a good transfection level *in vitro*.

Example 11: transfection *in vivo* of compound (1)

This example illustrates the capacity of compound (1) according to the invention to transfect DNA into cells *in vivo*, compared with nonformulated DNA and with lipid A having the condensed formula $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2COArgN[CH_2)_{17}CH_3]_2$ described in Application WO 97/18185 and whose structure is represented represented in Figure 1.

The gene transfer *in vivo* was performed on Balb/C mice by intramuscular and intravenous administration. The formulations which were compared are formulations of naked DNA, formulations containing

lipid A, or formulations containing compound (1) according to the invention.

In the case of intramuscular injections, each mouse received 30 µl of formulation containing 15 µg of DNA in the anterior muscle of the tibia. The tissues are recovered 7 days after the injection, they are frozen and stored at -80°C while waiting to perform the luciferase activity tests. The measurements of luciferase activity are carried out as in Example 8.

In the case of injections by the intravenous route, each mouse received 200 µl of formulation containing 50 µg of DNA. The tissues are recovered in this case 24 hours after the injection and then frozen and stored in the same manner as above.

The results of gene transfer *in vivo* are presented in Figure 8 and Figure 9. The ratio between compound (1) and the DNA is 10 nmol/µg of DNA. The ratio between lipid A and the DNA is 4 nmol/µg of DNA.

Figure 8 illustrates the *in vivo* activity in the muscle of compound (1) according to the invention compared with naked DNA and with lipid A. It is observed that the levels of luciferase activity are equivalent between naked DNA and compound (1), the latter having, in addition, a highly improved activity compared with lipid A. The transfer mechanisms involved appear to be different between naked DNA and the use of compound (1) according to the present invention. Indeed, the complexes according to the invention used

do not contain free DNA (phase C) and furthermore, their results *in vitro* are considerably greater than those for naked DNA.

Figure 9 compares the activity of compound (1) according to the invention, of naked DNA and of lipid A, by the intravenous route and by the intramuscular route.

It is observed that the transfection efficiency is roughly equivalent by the intravenous route for lipid A and for compound (1). On the other hand, by the intramuscular route, the transfection efficiency of compound (1) according to the invention is quite considerably greater than that of limid A.

Compared with naked DNA, compound (1) exhibits transfection by the intravenous route, in addition to transfection by the intramuscular route which is at least equivalent.

It therefore appears that the nucleic acid transfer efficiency *in vivo* with compound (1) according to the invention is greater overall than that with lipid A which is a known cationic lipid and that of nonformulated DNA.

Finally, it appears that the complexes according to the invention have the advantage, compared with transfection of naked DNA, of protecting the DNA from degradation by nucleases, thus contributing to a significant improvement in the stability of the formulations. The compounds of the present invention

can also be used to protect DNA from damage during freeze-drying, improving here again the stability of the formulations.

Example 12: transfection *in vivo* of compounds (5) and (6)

This example illustrates the capacity of compounds (5) and (6) to transfect nucleic acid *in vivo* in an efficient manner.

The same protocol as in the preceding example is used. Figure 10 shows that compound (5) and compound (6), formulated in a 0.25:1 charge ratio with DNA without co-lipid, exhibit a transfection level *in vivo* greater than or equal to naked DNA 48 hours after i.m. injection.

The following table gives the results obtained with compounds (5) and (6) in various formulations:

Compound	Compound/ DNA charge ratio	Co-lipid	RLU/ lung	pg/ lung	Route of adminis-tration
Compound (5)	6/1	DOPE (1:1)	254.9	1611.3	i.v.
Compound (5)	8/1	DOPE (1:1)	535.2	3558.1	i.v.
Compound (5)	0.5/1	Chol. (3:1)	209.6	1330.5	i.m.
Compound (5)	0.5/1	DOPE (1:1)	155.8	974.6	i.m.
Compound (6)	6/1	-	175.1	1098.7	i.v.
Compound (6)	5/1	DOPE (1:1)	407.7	2700.8	i.v.
Compound (6)	0.5/1	DOPE (1:1)	1768.7	13005.4	i.m.